Title of the Invention

A Live, Avirulent Strain of *V. anguillarum* that Protects Fish Against Infection by Virulent *V. anguillarum* and Method for Making the Same

Cross-Reference to Related Applications

This application claims the benefit of U.S. Provisional Application No. 60/220,733, filed July 26, 2000.

Statement Regarding Federally Sponsored Research or Development

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant No. 97352044811 awarded by the USDA.

Background of the Invention

1. Field of the Invention

The present invention relates to the identification, characterization and sequencing of a gene, the *mugA* gene, and to a live, attenuated strain of *V. anguillarum* used as a vaccine against vibriosis.

2. Description of the Related Art

Vibriosis is one of the major bacterial diseases affecting fish, bivalves and crustaceans in the marine environment. *Vibrio anguillarum*, a gram negative curved rod, is the causative agent of vibriosis. Symptoms of the disease in salmonids include red necrotic lesions in the abdominal muscle, erythema at the base of the fins, and subdermal hemorrhaging. The rectum becomes distended and filled with fluid, and hemorrhaging can often be observed in the internal organs. The bacteria become dispersed throughout the host tissues, including the kidney, liver and spleen. The highest concentration of bacteria is often found in the blood. Vibriosis typically

culminates in a hemorrhagic septicemia that causes infected stocks to suffer mortalities ranging from 30-100%. As a result, vibriosis is a significant limitation to aquaculture, causing large economic losses to the industry.

The fish gastrointestinal tract has been implicated as a site of colonization and growth of pathogenic *Vibrio* species. (Horne et al.; J. Fish Dis. 6: 461-471 (1983). It has been discovered that *Vibrio ordalii* and *Vibrio anguillarum* primarily infect the intestinal tracts and pyloric cecae of Pacific salmon. (Ransom et al.; J. Fish Dis. 7 107-115 (1984). It has been suggested that the fish gastrointestinal tract serves as a portal entry for *V. anguillarum*, and that the infection of the fish host begins with the colonization of the posterior gastrointestinal tract and the rectum. (Olsson et al.; J. Fish Dis. 19:225-234 (1996). It has been demonstrated that *V.anguillarum* exhibits chemotactic mobility towards intestinal mucus, and that motile *V. anguillarum* cells penetrate crude mucus preparations. Additionally, it has been demonstrated that *V. anguillarum* adheres to fish intestinal tissue and mucus and to the brush border cells of larval turbot. (Bordas et al.; Appl. Environ. Microbiol. 62:3650-3654 (1996)(Grisez et al.; Dis. Aquat. Org. 26: 181-187 (1996). Following infection of the gastrointestinal tract, it appears that *V. anguillarum* cells traverse the intestinal epithelium, enter the lamina propria, and spread systemically.

Gastrointestinal mucus is a rich nutrient source that many organisms, including pathogens, can utilize for growth. Studies suggest that growth in mucus is a critical factor to intestinal colonization of a host by pathogens, and that specific physiological changes occur in these bacteria in response to growth in mucus. (Burghoff et al.; Infect. Immun. 61:1293-1300 (1993)(Krivan et al.; Infect. Immun. 60: 3943-3946 (1992). It has been demonstrated that *V. anguillarum* grows rapidly and efficiently in salmon intestinal mucus. (Garica et al.; Appl. Environ. Microbiol. 63: 1034-1039 (1997). The cells typically exhibit a rapid generation time

and express at least five new membrane proteins in mucus-salts medium. Four of the proteins produced during growth in mucus are located in the outer membrane of the cell, while the fifth is located in the cytoplasmic membrane. (Garica et al.; Appl. Environ. Microbiol. 63: 1034-1039 (1997). The present invention provides vaccines and methods for making the same that can be used to prevent vibriosis infection in animals.

Brief Summary of the Invention

Broadly, this invention comprises a live, attenuated strain of *V. anguillarum* which comprises a mutated *mugA* gene and method for making the same. The strain is characterized in that it is incapable of expressing a functional *mugA* protein.

Further, the invention comprises a vaccine strain against *V. anguillarum* infection in an animal comprising a live, attenuated strain of *V.anguillarum* wherein the strain is comprised of a mutated *mugA* gene. The vaccine strain is characterized in that it is incapable of expressing a functional *mugA* protein.

Another aspect of the invention comprises the method of making and administering the vaccine strain. More particularly, the invention comprises the administration of the vaccine strain to animals selected from the group consisting of fish, bivalves and crustaceans.

In yet another aspect, the invention comprises administering the vaccine strain to animals by immersion, by intraperitoneal injection and oral or anal intubation.

The invention also comprises a method of inducing an immune response in an animal against one or more pathogens which comprises transforming a live, attenuated strain of *V. anguillarum* having a mutated *mugA* gene with a plasmid comprising DNA of interest encoding at least one protein antigen for each of the pathogens and administering the transformed strain to an animal.

In yet another aspect, the invention comprises the vaccine strains disclosed herein admixed with a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers can include water, the culture fluid in which the bacteria are cultured, a solution of physiological salt concentration and diluents selected from the group consisting of stabilizers, carbohydrates, proteins and protein containing agents.

Another aspect of the invention comprises the identification, characterization and sequencing of the *mugA* gene and the amino acid sequence of the protein encoded by the *mugA* gene.

In yet another aspect, the invention comprises the use of the *mugA* gene as a DNA probe to detect the presence of *V. anguillarum* in animals or media. More particularly, the invention relates to use of the *mugA* gene as a DNA probe to detect the presence of *V. anguillarum* in animals selected from the group consisting of fish, bivalves and crustaceans and media selected from the group consisting of sediment and water.

The invention also comprises DNA primers constructed from the *mugA* gene sequence to be used in polymerase chain reaction (PCR) or reverse transcriptase (RT) or a combination of RT and PCR to detect the presence of *V.anguillarum* cells, DNA or RNA.

Also within the scope of this invention is the use of the protein encoded by the *mugA* gene to construct protein subunit vaccines against *V.anguillarum* infection in animals. Further, the invention comprises the use of antibodies against the *mugA* protein for the development of passive vaccines against *V. anguillarum* infection, the treatment of *V. anguillarum* infection in animals and the development of immunodiagnostic reagents to detect the presence of *V. anguillarum* in animals or media.

Brief Description of the Drawing(s)

Fig. 1 is a gene map of the cloned region of DNA containing *mugA* which depicts the nucleotide lengths of the complete open reading frames indicated underneath.

Fig. 2 depicts the nucleotide sequence of the cloned region of DNA containing *mugA*.

Fig. 3a depicts the putative amino acid sequence of ORF A/MugA.

Fig. 3b depicts the putative amino acid sequence of ORF B.

Fig. 3c depicts the putative amino acid sequence of ORF C/EiaA.

Description of the Preferred Embodiment(s)

The invention will be described with reference to following non-limiting examples.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids, and preparation of mucus.

The bacterial strains used are listed in Table 1. *Vibrio anguillarum* M93 (Mitsuru Eguchi, Department of Fisheries, Kinki University, Nara, Japan) is serotype J-O-1. All *V. anguillarum* strains were routinely grown in Luria-Bertani broth + 2% NaCl (LB20) supplemented with the appropriate antibiotic, on a rotary shaker at 27°C. Experimental media included: LB20, Marine Minimal Medium (3M), and nine salts solution (NSS) supplemented with 200 μg salmon gastrointestinal mucus protein/ml (NSSM). Garcia et al.; Growth of *Vibrio anguillarum* in salmon intestinal mucus. Appl. Environ. Microbiol. 63: 1034-1039 (1997). All *Escherichia coli* strains were routinely grown in Luria-Bertani broth + 1% NaCl (LB10), supplemented with the appropriate antibiotic. *E. coli* CC118 is a lambda lysogen carrying the *pir* gene required for replication of pLOFKm. The plasmid pLOFKm is a Tn*10*-based delivery plasmid with kanamycin resistance. Ausubel et al.; Current Protocols in Molecular Biology (1987). Herrero et al.; Transposon vectors containing non-antibiotic resistance selection markers

for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. 172: 6557-6567 (1990). The plasmid pBluescript II SK+ (Stratagene, La Jolla, CA) was used as a cloning vector, and pTGDN14 and subclones were pBluescript derivatives. Salmon gastrointestinal mucus was prepared as previously described. Garcia et al.; Growth of *Vibrio anguillarum* in salmon intestinal mucus. Appl. Environ. Microbiol. 63: 1034-1039 (1997). Cell densities for all experiments were determined by serial dilution and plating on LB20 agar plates. Antibiotics were used at the following concentrations for *V. anguillarum*: streptomycin, 200 μg/ml (Sm^{200}); kanamycin, 85 μg/ml (Km^{85}). Antibiotics were used at the following concentrations for *E. coli*: kanamycin, 40 μg/ml (Km^{40}); ampicillin, 100 μg/ml (Ap^{100}).

Selection of a streptomycin-resistant mutant of *V. anguillarum*.

A streptomycin-resistant mutant of *V. anguillarum* M93 was selected by spread plating 100 μl of an overnight culture onto LB20 agar plates supplemented with 100 μg/ml streptomycin. Streptomycin resistant mutants able to grow in media containing 100 μg/ml streptomycin were transferred to LB20 + 200 μg/ml streptomycin. Mutants able to grow in LB20 + 200 μg/ml streptomycin were tested for the ability to grow in LB20 and NSSM. A streptomycin-resistant mutant (*V. anguillarum* M93Sm) that exhibited growth rates similar to the parental wild-type strain was selected for further use. Denkin et al.; Induction of protease activity in *Vibrio anguillarum* by gastrointestinal mucus. Appl. Environ. Microbiol. 65: 3555-3560 (1999).

Transposon insertion mutagenesis of *Vibrio anguillarum* M93Sm.

Transposon insertion mutagenesis was employed to create *V. anguillarum* M93Sm mutants, using the mini-Tn*10* based transposon vector delivery system developed by Herrero et al. Herrero et al.; Transposon vectors containing non-antibiotic resistance selection markers for

cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. 172: 6557-6567 (1990) Briefly, *V. anguillarum* M93Sm was conjugated with *E. coli* CC118 (λ*pir*)(pLOFKm), which contains the mini-Tn*10* (Km^r). Aliquots (100 µl) from overnight cultures of each organism were mixed in 2.5 ml NSS plus 2.5 ml 10 mM $MgSO_4$. The suspension of cells was vacuum filtered onto a 0.22 µ filter. The filter was placed on an LB15 agar plate (Luria-Bertani agar + 1.5% NaCl) and allowed to incubate overnight at 27°C. After incubation the cells on the filter were resuspended in 2.5 ml NSS plus 2.5 ml 10 mM $MgSO_4$, and 100 µl of the cell suspension was spread plated onto LB20 Sm^{200} Km^{85} agar plates and allowed to incubate overnight at 27°C.

Selection of mucus growth mutants.

Colonies derived from the mutagenesis procedure were scraped from the LB20 Sm^{200} Km^{85} agar plates and pooled in 10 ml of NSS. Ten microliters of the *V. anguillarum* cell suspension was added to a carbenicillin counter-selection medium containing 50 ml NSS supplemented with 300 µg/ml mucus protein plus streptomycin (200 µg/ml), kanamycin (85 µg/ml) and carbenicillin (250 µg/ml). Cells were incubated in this counter selection medium at 27°C for 3 h. After incubation, the cells were removed from the counter-selection medium by centrifugation (12,000 x g, 30 min). The cells were washed twice in NSS, resuspended in 50 ml of LB20 plus Sm^{200} Km^{85} and allowed to grow overnight on a rotary shaker at 27°C. After growth, the cells were put through two more rounds of carbenicillin counter-selection. After the third round of selection, cells were washed free of the counter-selection medium and 100 µl aliquots were spread onto LB20 $Sm^{200}Km^{85}$ agar plates. Isolated colonies were screened on LB20 $Sm^{200}Km^{85}$ plates and mucus agar $Sm^{200}Km^{85}$ plates (300 µg/ml mucus protein). Colonies that did not grow on mucus agar plates were retained.

Southern DNA transfer, hybridization analysis and DNA probes.

For all Southern analysis, total genomic DNA was extracted from bacteria and digested to completion with *PstI* (Promega, Madison, WI). The resulting fragments were loaded in equal amounts on an agarose gel and were separated by agarose gel electrophoresis (0.8% agarose gel, 70 V) in Tris acetate buffer. DNA samples were transferred from the agarose gel to a nylon membrane (MagnaGraph, MSI, Westboro, MA) for Southern hybridization analysis (15 h hybridization at 60°C). Ausubel et al.; Current Protocols in Molecular Biology (1987).

To confirm the mini-Tn*10* insertion in *V. anguillarum* M93Sm chromosomal DNA, blots were probed with a digoxigenin-dUTP labeled kanamycin cassette. Briefly, the kanamycin cassette probe was constructed by digesting the pUC4K plasmid (Pharmacia Biotech, Piscataway, NJ) with *Pst*I. The resulting fragments were separated by agarose gel electrophoresis, and the 1.24 kb kanamycin cassette was purified from the agarose gel using a Geneclean Spin Kit (Bio 101, Vista, CA). The kanamycin cassette was then labeled with digoxigenin-dUTP using a DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim, Germany) according to the instructions of the manufacturer.

To screen for the presence of the *mug*A gene in *V. anguillarum* and other bacteria, blots were probed with a digoxigenin-dUTP labeled *mug*A gene probe. Briefly, primers were derived from the sequence of the mini-Tn*10* interrupted gene as *mugA*-forward (5'-TTTCTGCAGCTGGTTGAAATAACTCAAGGCC-3') and *mugA*-reverse (5'-TTTCTGCAGGGATCCGAAACGGAAGGCTTCGC-3') (Gibco BRL). A 1.4 kb DNA fragment of the *mugA* gene was PCR amplified from *V. anguillarum* genomic DNA using the primers and a PCR-DIG Probe Synthesis Kit (Boehringer Mannheim, Germany) according to the instructions of the manufacturer. PCR conditions are indicated below.

Cloning and mapping of the *mugA*::mini-Tn*10* region of DNA.

Initially, the gene fragment containing the mini-Tn*10* *mugA* was cloned and sequenced. Total genomic DNA was extracted from *V. anguillarum* M93SmΩD and digested to completion with *Pst*I. The cloning vector pBluescript (Stratagene) was digested with *Pst*I (Promega, Madison, WI), treated with calf intestinal phosphatase (Promega, Madison, WI), and ligated (1 U/ μl DNA ligase; Promega, Madison, WI) to the *Pst*I digested genomic DNA. Plasmid DNA containing genomic DNA was then transformed into the competent *E. coli* host (XLI MRF', Km^S). Ausubel, F. M. et al.; Current Protocols in Molecular Biology (1987). Transformants were screened for the presence of the transposon-interrupted gene by plating onto LB Km^{40} plates. Plasmid DNA of transformants exhibiting kanamycin resistance was extracted by pelleting 1-3 ml *E. coli* cells (~2.0 x 10^9 cells/ml), resuspending the cell pellet in 200 μl Cell Resuspension Solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 μg/ml RNAse A), lysing the cells in 200 μl Cell Lysis Solution (0.2 M NaOH, 1% SDS), neutralizing the lysate with Neutralization Solution (1.32 M potassium acetate, pH 4.8) and isolating plasmid DNA using a Wizard Minicolumn (Wizard Mini-Preps kit, Promega, Madison, WI). The cloned mini-Tn*10* interrupted gene was mapped by digesting the cloned DNA with various restriction enzymes followed by electrophoresis and analysis of the resulting fragments on a 0.8% agarose gel. Restriction enzymes used for mapping include *Pst*I, *Eco*RI, *Eco*RV, *Bam*HI, *Hind*III, *Sal*I, *Sac*I, *Spe*I, *Hinc*II, *Cla*I, *Kpn*I *Sma*I, *Xho*I, *Bal*I, *Bst*XI, *Not*I, *Xba*I, and *Sty*I (Promega, Madison, WI). Subcloning was performed as described in the Results section of this report to obtain smaller DNA fragments for DNA sequencing.

To obtain the wild-type *mugA* gene, total genomic DNA of *V. anguillarum* M93Sm was extracted and digested to completion with *Hind*III (Promega) and *Bam*HI (Promega), ligated to

*Hind*III and *Bam*HI digested pBluescript, and transformed into *E. coli* (XLI MRF'). Tranformants were plated on LB10 Amp^{100} plates containing isopropylthiogalactoside (IPTG; 100 mM) and 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 80 μg/ml) (BIO-RAD laboratories, Richmond, CA) and allowed to incubate overnight at 37°C. Plating on this medium allowed for blue (lac^+) - white (lac^-) screening for inserts in the *lac*Z gene of pBluescript. White ampicillin resistant colonies were transferred to fresh LB10 Amp^{100} plates, allowed to grow overnight at 37°C, and blotted onto nylon membranes. Colony blots were performed as previously described (4), using the digoxigenin-dUTP labeled *mugA* gene as a probe. Colonies that hybridized to the *mugA* probe were screened by PCR analysis, using the *mugA* forward and reverse primers described above. DNA sequence analysis revealed that this yielded a 2 kb fragment containing all but the last 113 bp of the *mugA* gene. The entire wild-type *mugA* gene was obtained following PCR amplification from *V. anguillarum* M93Sm using the *mugA*-forward primer (described above) and *mugA*-reverse2 primer (5'-TTTAAGCTTCACGCATGTAAATACTTGCC-3').

PCR conditions and DNA Sequencing.

The same conditions were employed for all genomic and plasmid DNA samples to be amplified by PCR. All samples were amplified using *Taq* polymerase (3.5 U/100 μl reaction mixture; Gibco BRL Life Technologies, Bethesda, MD) on a Perkin Elmer GeneAmp Model 9600 Thermocycler (Perkin Elmer Cetus, Norwalk, CT). PCR cycle conditions were: 94°C, 1 min; 51°C, 2 min; 72°C, 3 min. The reaction was run for 35 cycles and then held at 4°C until collected. All DNA sequencing was performed by the W.M. Keck Laboratory at Yale University, New Haven, CT. The DNA was sequenced using *Taq* FS DNA polymerase and

fluorescent-dideoxy terminators in a cycle sequencing method and the resultant DNA fragments were electophoresed and analyzed using an automated Applied Biosystems 377 DNA sequencer.

Fish infections.

All fish infection experiments were carried out for a period of 21 d. *V. anguillarum* cells were prepared overnight by culture in LB20 overnight with shaking (27°C) to a density of ~2 x 10^9 CFU/ml. Cells were harvested by centrifugation, washed twice in NSS, resuspended in NSS, and diluted to the appropriate inoculation concentrations. Prior to inoculation, Juvenile Atlantic salmon (*Salmo salar*) were anesthetized in water supplemented with tricaine methane sulfonate (75g/L). Fish were injected intraperitoneally with equal volumes (50 µl) of either NSS alone (control fish) or cells resuspended in NSS. Plate counts were performed to determine the actual number of cells injected. Fish were maintained in 2 m diameter, 200 liter tanks partially submerged in flowing water to maintain tank temperatures at ~15°C throughout the experiment. Death due to vibriosis was determined by the observation of gross clinical signs (including, but not limited to, observation of petechiae, hemorrhaging through the vent, presence of lesions in the walls of the abdomen) of the disease and recovery of vibrio (*V. anguillarum* M93) or streptomycin resistant vibrio (*V. anguillarum* M93Sm) or streptomycin and kanamycin resistant vibrio (*V. anguillarum* M93SmΩD) from dead fish. Challenge of fish surviving *V. anguillarum* M93SmΩD infection commenced 7 d after conclusion of the initial fish infection experiment and was performed using the same procedure, under the previously described conditions.

Computer analysis of DNA and amino acid sequences.

Open reading frames were determined from the nucleotide sequence using ORF Finder. www.ncbi.nlm.nih.gov/gorf/gorf.html.;Altschul et al.; Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402 (1997).

Putative promoters were determined using Promoter Prediction. Reese, M. G.;Diploma Thesis. German Cancer Research Center, Heidelberg, Germany (1994); Reese et al; Novel Neural Network Algorithms for Improved Eukaryotic Promoter Site Recognition. The Seventh International Genome Sequencing and Analysis Conference, Hilton Head Island, South Carolina (1995); Reese et al.; Large Scale Sequencing Specific Neural Networks for Promoter and Splice Site Recognition. Biocomputing: Proceedings of the 1996 Pacific Symposium. World Scientific Publishing Co., Singapore (1996). BLASTn and BLASTp searches were performed to determine nucleotide and protein sequence similarities. Altschul et al.; Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402. (1997) Probable subcellular localization of putative proteins was determined using PSORT. Nakai et al; Expert system for prediction protein localization sites in Gram-negative bacteria, PROTEINS: Structure, Function, and Genetics. 11: 95-110 (1991). Database searches for amino acid composition similarities were performed using Propsearch and AACompIdent Tool (ExPASy Server). Hobohm, et al.; A sequence property approach to searching protein databases. J. Mol. Biol. 251: 390-399 (1995); Wilkins et al.; Protein Identification and Analysis Tools in the ExPASy Server in: 2-D Proteome Analysis Protocols. (1998).

RESULTS

Growth of *V. anguillarum* M93Sm and M93SmΩD in LB20 and mucus medium.

The procedure for the construction and selection of mini-Tn*10* insertion mutants of *V. anguillarum* M93Sm unable to grow on mucus agar plates was performed as described in the Materials and Methods. One insertion mutant, *V. anguillarum* M93SmΩD, which grew on LB20 Sm^{200} Km^{85} agar and exhibited no growth on mucus agar plates (300 μg/ml mucus protein) was selected and used. Referring to Table 2, the growth of M93SmΩD was observed in LB20 and

NSSM and compared to the growth of M93Sm in identical media. *V. anguillarum* M93Sm and M93SmΩD grew to comparable levels in LB20 after 24 h (~4-6 x 10^9 CFU/ml), with CFU increases of about 1600 fold and 1900 fold, respectively. In NSSM, M93Sm grew to a maximum CFU of >4.6 x 10^9 CFU/ml in 10 h and subsequently declined to ~1.4 x 10^9 CFU/ml by 24 h. This decline in viability following growth in mucus was previously reported by Garcia et al. Garcia et al.; Growth of *Vibrio anguillarum* in salmon intestinal mucus. Appl. Environ. Microbiol. 63: 1034-1039 (1997). In contrast, M93SmΩD failed to grow in mucus over a 24 h incubation. The cell density declined 18-fold from 2.13 x 10^6 CFU/ml to 1.16 x 10^5 CFU/ml.

Determination of mini-Tn*10* insertion by Southern analysis.

In order to confirm that the *V. anguillarum* M93SmΩD genome contained a mini-Tn*10* insertion, *Pst*I digested genomic DNA from *V. anguillarum* M93Sm and M93SmΩD were each probed with a dUTP-digoxigenin labeled kanamycin cassette. Unlabeled kanamycin cassette (1.3 kb) was hybridized with the probe as a control. Southern analysis of the genomic DNA revealed a hybridizable 11 kb fragment in M93SmΩD. No hybridizable fragments were observed in the M93Sm genomic DNA. The results demonstrate that the transposon was present in the M93SmΩD genomic DNA as a single copy.

Cloning, subcloning, and sequencing of the transposon-interrupted gene.

Since Southern analysis had shown that the mini-Tn*10* insertion was contained within an 11 kbp fragment, *PstI* digested DNA from *V. anguillarum* M93SmΩD was ligated with *PstI*-digested pBluescript SKII and transformed into *E. coli* XLI MRF'. Transformants were selected on LB10 Amp^{100} Km^{40} plates. Restriction endonuclease analysis of a pBluescript-derived plasmid conferring kanamycin resistance demonstrated that the plasmid contained an 11 kbp *Pst*I-*Pst*I fragment (pTGDN14).

In order to obtain smaller fragments for sequencing, this plasmid was subjected to further restriction endonuclease analysis and subcloning. Referring to Figs. 1, 2, and 3a-c, the plasmid pTGDN14 was digested with *Sal*I and *Sac*I, and a 3.1 kb fragment of DNA containing the mini-Tn*10* insert was isolated and ligated to *Sal*I and *Sac*I digested pBluescript, yielding pTGDN6. Additionally, pTGDN14 was digested with *Sal*I and religated, to eliminate a 4.2 kbp *Sal*I-*Sal*I fragment of *V. anguillarum* DNA. The resulting subclone was termed pTGDN10. The pTGDN10 construct consisted of 7 kbp of DNA containing the mini-Tn*10* insertion in a *Sal*I-*Pst*I fragment ligated to pBluescript. Sequencing was performed by primer walking from the T7 promoter to the mini-Tn*10* insert on pTGDN6, and from the mini-Tn*10* insert on pTGDN10 outwards toward the T3 promoter. A map and sequence of the region of DNA containing the mini-Tn*10* insert is presented in Figure 1. The regions of the two subclones that were sequenced are indicated. The putative amino acid sequences of the three ORFs are shown in Figs. 3a-c. Open reading frames depicted in Fig. 1 are indicated as shaded boxes, with nucleotide lengths for complete ORFs indicated underneath. Directions of transcription are indicated by horizontal arrows. The transposon insertion site is indicated by a vertical arrow. Subclones utilized for sequencing are indicated. The entire 3.59 kb nucleotide sequence is depicted in Fig. 2. Proposed promoter sites are underlined and indicated with a curved arrow. Putative Shine-Delgarno sequences are labeled (S-D) and underlined. Start codons for each ORF are underlined and indicated by horizontal arrows followed by the ORF name. Stop codons are underlined and indicated with an asterisk. The sequence of ORF C is incomplete and no stop codon is indicated. The transposon insertion site is indicated with a bold vertical arrow.

The entire wild-type *mugA* gene was obtained following PCR amplification from *V. anguillarum* M93Sm using the *mugA*-forward primer and *mugA*-reverse2 primer. Utilization of

the *mugA*-reverse2 primer allowed PCR amplification of the entire *mugA* gene, including the last 113 bp of the *mugA* gene, which is not amplified when using the *mugA*-reverse1 primer. Thus, while the PCR product generated using the *mugA*-forward and *mugA*-reverse1 primers is ~1.4 kb, the PCR product generated using the *mugA*-forward and *mugA*-reverse2 primers is approximately 1.5 kb.

Screening of chromosomal DNA from various bacteria using *mug*A as a DNA probe.

Southern hybridization analysis was employed to determine whether the *mugA* gene was present in bacteria other than *V. anguillarum* M93. Genomic DNA from *V. anguillarum* strains M93Sm, NB10 and 2129 and *E. coli* ATCC 25922 was probed with the *mug*A DNA probe. Milton et al.; Flagellin A is essential for the virulence of *Vibrio anguillarum*. J. Bacteriol. 178: 1310-1319 (1996); Garcia et al.; Growth of *Vibrio anguillarum* in salmon intestinal mucus. Appl. Environ. Microbiol. 63: 1034-1039 (1997). Unlabeled *mug*A (1.4 kb) was hybridized with the probe as a control. All strains of *V. anguillarum* tested showed a single band of hybridizing DNA at approximately 9.5 kbp. No hybridizing DNA was observed in *E. coli* ATCC 25922, nor were hybridizing bands observed when DNA from *Aeromonas hydrophila*, *A. salmonicida*, *V. carcariae*, and *V. parahemolyticus* were probed with *mugA*.

Fish infection studies.

As depicted in Table 3, the virulence of *V. anguillarum* wild type (M93) and mutant strains (M93Sm and M93SmΩD) were compared by injecting various concentrations of cells intraperitoneally into Atlantic salmon (Table 3). The virulence of *V. anguillarum* M93 (wild type) was compared to M93Sm (Sm^r mutant) to determine whether the mutation conferring streptomycin resistance affected virulence. No mortalities were observed in fish injected with NSS alone. All fish injected with 10^6, 10^7 and 10^8 *V. anguillarum* M93 and M93Sm cells died in

1-3 days. These data demonstrated that the virulence of *V. anguillarum* M93 and the streptomycin-resistant derivative *V. anguillarum* M93Sm were similar. In contrast, no fish died following injection with *V. anguillarum* M93SmΩD at any of the doses administered.

As depicted in Table 4, to determine whether injection with *V. anguillarum* M93SmΩD provided protection to fish against vibriosis caused by a virulent strain, Atlantic salmon that survived injection with *V. anguillarum* M93SmΩD were challenged with lethal doses of *V. anguillarum* M93Sm (Table 4). The unvaccinated (control) fish were from the same stock and were the same age as the vaccinated fish. All unvaccinated control fish inoculated with *V. anguillarum* M93Sm at concentrations of 10^6 and 10^5 cells died in 2 days and 4 days, respectively. One of five fish died in 4 days when inoculated with 10^4 cells. In contrast, no vaccinated fish died following injection of 10^6 and 10^5 *V. anguillarum* M93Sm cells. These data demonstrate that prior inoculation of Atlantic salmon with the avirulent *V. anguillarum* M93SmΩD is protective against vibriosis caused by the virulent M93Sm.

Table 1. Bacterial strains.

Species and Strains	Description
V. anguillarum	
M93	wild type
M93Sm	streptomycin-resistant (Sm^r) mutant of M93
M93SmΩD	M93 (*mug*A::Tn*10*), Sm^r Km^r
NB10	wild type
2129	wild type
E. coli	
CC118	(*Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 RpsE rpoB argE(Am) recA1*)
XLI MRF'	cloning vector host strain
ATCC 25922	wild type
A. hydrophila	wild type
A. salmonicida	wild type

V. carcariae	wild type
V. parahemolyticus	wild type

Table 2. Growth of *Vibrio anguillarum* M93Sm and M93SmΩD in LB20 and NSSM (200 μg mucus)[a]

Time (h)	CFU/ml LB20		NSSM	
	M93Sm	M93SmΩD	M93Sm	M93 SmΩD
0	3.73 (± 0.25) x 10^6	2.03 (± 0.32) x 10^6	2.53 (± 0.31) x 10^6	2.13 (± 0.21) x 10^6
10	4.67 (± 0.37) x 10^9	1.40 (± 0.35) x 10^9	4.67 (± 1.50) x 10^9	4.73 (± 0.45) x 10^5
24	6.00 (± 0.20) x 10^9	3.90 (± 0.46) x 10^9	1.43 (± 0.12) x 10^9	1.16 (± 056) x 10^5
Fold Change (0-24h)	1596 increase	1921 increase	565 increase	18 decrease

[a]Cells were grown overnight in LB20 (27°C) with shaking and prepared for inoculation into LB20 or NSSM as described in the Materials and Methods section. Cell density was determined by serial dilution of samples and plating onto LB20 agar as described in the Materials and Methods section. All cell density determinations were done in triplicate.

Table 3. Comparison of virulence of *V. anguillarum* M93, *V. anguillarum* M93Sm, and *V. anguillarum* M93SmΩD in Atlantic salmon [a]

Strain	Approximate Dose/fish (CFU)	Mortality	Time of death due to vibriosis
V. anguillarum M93	10^6	4/4	1 d
	10^7	5/5	1 d
	10^8	5/5	1 d (4/5), 2 d (5/5)
V. anguillarum M93Sm	10^6	4/4	2 d (3/4), 3 d (4/4)
	10^7	5/5	1 d
	10^8	5/5	1 d
V. anguillarum M93SmSD	10^6	0/4	NA
	10^7	0/5	NA
	10^8	0/5	NA

[a] *V. anguillarum* cells were prepared by growing in LB20 overnight (27°C) to a density of 2 x 10^9 CFU/ml. Cells were harvested by centrifugation, washed twice in NSS, resuspended in NSS, and diluted to the appropriate inoculation concentrations. All fish were injected IP with equal volumes (50 µl) of either NSS alone (control fish) or cells resuspended in NSS. Plated counts were performed to determine the actual number of cells injected. The experiment continued for 21 d. No mortalities were observed in control fish throughout the 21 d period. Death due to vibriosis was determined by examination of clinical signs and recovery of vibrio (*V. anguillarum* M93), streptomycin resistant vibrio (*V. anguillarum* M93Sm) or streptomycin and kanamycin resistant vibrio (*V. anguillarum* M93SmΩD).

Table 4. Challenge of *V. anguillarum* M93SmΩD infection survivors with lethal doses of *V. anguillarum* M93Sm [a]

Fish	Approximate Dose/fish (CFU)	Mortality	Time of death due to vibriosis
Unvaccinated fish [b]	10^4	1/5	4 d
	10^5	5/5	4 d
	10^6	5/5	2 d
Vaccinated fish	10^5	0/5	NA
	10^6	0/5	NA

[a] *V. anguillarum* cells were prepared by growing in LB20 overnight (27°C) to a density of 2 x 10^9 CFU/ml. Cells were harvested by centrifugation, washed twice in NSS, resuspended in NSS, and diluted to the appropriate inoculation concentrations. All fish were injected IP with equal volumes (50 µl) of either NSS alone (control fish) or cells resuspended in NSS. Plated counts were performed to determine the actual number of cells injected. The experiment continued for 21 d. No mortalities were observed in control fish throughout the 21 d period. Death due to vibriosis was determined by examination of clinical signs and recovery of streptomycin resistant vibrio (*V. anguillarum* M93Sm).

[b]Unvaccinated control fish are from the same stock and are the same age as the vaccinated fish.

DISCUSSION

Both the region of DNA containing the transposon insertion and a wild type version of that region were cloned and sequenced. Approximately 2.5 kbp of the sequenced region contained 3 ORFs. The ORF encoding *mug*A is 1.39 kbp in length and encodes a putative protein of 51.6 kDa (463 amino acids) with a pI of 5.24. Referring to Fig. 2, a proposed promoter site and a ribosomal binding site was located 146 bp and 5 bp upstream from the ATG start codon of the *mug*A gene. Milton et al.; Flagellin A is essential for the virulence of *Vibrio anguillarum*. J. Bacteriol. 178: 1310-1319 (1996); Reese, M. G.; Diploma Thesis. German Cancer Research Center, Heidelberg, Germany (1994); Reese et al.; Novel Neural Network Algorithms for Improved Eukaryotic Promoter Site Recognition. The Seventh International Genome Sequencing and Analysis Conference, Hilton Head Island, South Carolina (1995);

Reese et al.; Large Scale Sequencing Specific Neural Networks for Promoter and Splice Site Recognition. Biocomputing: Proceedings of the 1996 Pacific Symposium. World Scientific Publishing Co., Singapore (1996). The nucleotide sequence shows no significant homology to any known sequence in Genbank. The amino acid sequence shows homology to *Enterococcus faecium* aggregation substance (27% identity, 42% similarity) in 109 amino acids toward the C-terminal end of MugA, and to myosin from several organisms, the most significant of which was from human skeletal muscle (22% identity, 38% similarity) over 319 amino acid residues. However, the MugA amino acid sequence demonstrated no significant homology to other bacterial proteins over the entire sequence.

The *mug*A gene probe only hybridized with DNA from strains of *V. anguillarum*, including M93, 2129 and NB10. Additionally, the *PstI* fragments with which it hybridized were approximately the same size in all strains (~9.5 kb). No hybridizing DNA fragments were found in several other fish pathogens tested, including *V. carchariae*, *V. parahemolyticus*, *A. salmonicida*, and *A. hydrophila*.

A second open reading frame, ORF B, is 530 bp in length and has an ATG start codon 16 bp immediately downstream of the *mug*A stop codon. A predicted ribosomal binding site is located 7 bp upstream from the start codon of ORF B. This ORF encodes for a putative protein of 18.6 kDa (176 amino acids) with a pI of 4.17. Database searches using the nucleotide and amino acid sequence show no significant homology to known sequences in GeneBank. Additionally, no similarities to other protein families were found when a database search was conducted based on amino acid composition.

A third open reading frame, ORF C, is located 144 bp downstream of ORF B. A putative promoter and a ribosome binding site are located at 23 bp and 8 bp upstream from the ATG start

codon, respectively. A database search using the incomplete nucleotide sequence (352 bp) showed no significant homology to known nucleotide sequences in Genbank. However, the amino acid sequence exhibits very strong similarity to a putative 19.5 kDa protein of unknown function from the fish pathogen *Edwardsiella ictaluri* (52% identity, 67% similarity), the causative agent of the disease enteric septicemia of catfish. ORF C has been termed *eiaA* (*E. ictaluri*-like antigen A). The ORF C amino acid sequence also showed strong similarity to an unknown hypothetical protein from *E. coli* (30% identity, 56% similarity). Database searches using the combined attributes of amino acid composition, molecular weight and pI found no similarities to other protein families.

While *V. anguillarum* M93 and M93Sm are highly virulent strains causing 100% mortality at doses of 10^5 CFU/fish, M93SmΩD is avirulent even when 10^8 CFU are injected IP (Tables 2 and 3). An equivalent dose (10^8 CFU) of *V. anguillarum* M93Sm killed all fish within 1 day. No mortalities and no evidence of vibriosis were observed over a 21 d period in fish injected with M93SmΩD. While IP injection is an artificial route of infection, these data demonstrate that the ability of *V. anguillarum* to grow in mucus affects the virulence of this organism. Additionally, fish previously injected with *V. anguillarum* M93SmΩD were completely protected against vibriosis when challenged with lethal doses (10^5 and 10^6 cells injected/fish) of the virulent *V. anguillarum* M93Sm. All fish that were not previously inoculated with M93SmΩD died 2-4 days after challenge at these doses.

Typical procedures for the construction of a subunit vaccine using *mugA*, the purification of anti-MugA antibodies, the utilization of anti-MugA antibodies for immunodetection of *V. anguillarum* and the passive immunization of fish using anti-MugA antibodies follow. Such procedures are well within the skill of the art.

Construction of a subunit vaccine using *mugA*

Clone *mugA* gene into an inducible expression vector to construct a gene/protein fusion between *mugA* and a portion of another gene, which permits rapid purification of the *mugA* protein (MugA). Examples of such vectors include: Glutathione S-transferase (GST) gene fusion vectors (e.g. pGEX vectors – available from Amersham Pharmacia Biotech) and calmodulin-binding peptide (CBP) vectors (e.g. pCAL vectors – available from Stratagene). The fusion proteins generated with these vectors allow the target protein to be purified by an affinity column using the fusion protein tag (GST or CBP) to bind to glutathione or calmodulin, respectively. Specific proteases are available to remove the protein purification tag from *mugA*. The proteases used to remove the protein purification tag include thrombin, Factor Xa, enterokinase protease, and prescission protease. The purified MugA protein can be mixed with an adjuvant to improve the antigenic response of the immunized animal. Adjuvants used in fish include Alum [$Al(OH)_3$] and mineral oil. The vaccine can be administered to the fish by injection.

Purification of anti-MugA antibodies

MugA, purified as described above and combined with an adjuvant as described above, will be injected into rabbits. The rabbits can be injected three times at two-week intervals to boost antibody production. Similar methods may be used to immunize other animals (i.e. goats, fish, etc.). Two weeks after the third injection, blood from the rabbits (or other immunized animals) can be collected. The antibody containing serum can be separated from the blood cells by low speed centrifugation and antibodies purified from the serum using affinity column chromatography employing either Sepharose-Protein A or -Protein G to bind the antibodies. Affinity purified antibodies can be further purified to yield anti-MugA antibodies by affinity

binding to Sepharose-MugA columns. Sepharose-MugA is created by reacting purified MugA protein with cyanogen bromide activated Sepharose.

Utilization of anti-MugA antibodies for immunodetection of *V. anguillarum*

Samples (tissue or water) containing suspected *V. anguillarum* can be reacted with anti-MugA antibodies. After a series of washes to remove any unreacted primary antibody, a second antibody reactive against rabbit antibodies (e.g. goat-anti-rabbit IgG) tagged with a detection reagent (e.g. FITC or alkaline phosphatase) can be added to the primary antibody-sample mixture. After reaction and washes to remove unreacted secondary antibody, the presence of MugA can be detected using the specific detection reagent on the secondary antibody.

Passive immunization of fish using anti-MugA antibodies

Animals to be passively immunized against *V. anguillarum* infection can be injected with a suspension of purified anti-MugA antibody. For best results, the antibodies can be made and purified from the same species as that being passively immunized. The use of same species antibody prevents immune reactions against the immunizing antibodies by the target animals.

All journal articles and reference citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference.

The foregoing description has been limited to a specific embodiment of the invention. It will be apparent, however, that variations and modifications can be made to the invention, with the attainment of some or all of the advantages of the invention. Therefore, it is the object of the appended claims to cover all such variations and modifications as come within the true spirit and scope of the invention.

Having described our invention, what I now claim is: